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Research Paper

Genistein enhances expression of extracellular regulated kinases (ERK) 1/2, and learning and memory of mouse

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ABSTRACT

Genistein (GEN) is a well known phytoestrogen. It acts through estrogen receptor (ER) and performs plethora of functions in the brain. ERK1/2 is an activated kinase which involves in neuron differentiation, adult neurogenesis and several brain functions including learning and memory. However, GEN dependent expression of ERK1/2 and its effect in learning and memory of mice are unknown. In this study, *Swiss albino* male mice of 25weeks weighing 30 g were used for the experiments. Mice were placed in two groups- control (C) and genistein treated (GEN). Treated group received GEN dissolved in sesame oil (1 mg/kg/day) whereas the control group received sesame oil only. To study the effects of GEN on learning and memory, open-field (OF) test and novel object recognition (NOR) test were performed. Moreover, immunoblotting (IB) was performed to check the expression of ERK1/2 in the mouse brain of both groups. In the OF test, no significant change was observed in motor activity and anxiety in GEN treated mice as compared to control. Moreover, NOR test suggested that entry towards the dissimilar object was higher in case of GEN treated mice as compared to control. These findings suggest higher learning and memory of GEN treated mice than of control. IB showed that the expression of ERK1/2 was significantly high in GEN treated mouse brain as compared to control. Such study may be helpful to understand GEN mediated learning and memory involving ERK1/2.

Introduction

Genistein (GEN) is a naturally occurring estrogenic substance mainly found in soybean. It shows structural and functional similarities with 17β-estradiol. With these similarities, GEN binds with estrogen receptors (ER). GEN shows different binding affinity towards ERα and ERβ. Its binding affinity towards ERβ is seven to eight times higher as compared to ERα (File et al., 2001; Tzagarakis-Foster et al., 2001). ERβ is abundantly present in the brain regions associated with learning and memory *namely* neocortex, hippocampus and nuclei of the basal forebrain (Shughrue et al., 1997; Wang et al., 2001; Xu et al., 2009). Earlier study suggested that the high dietary intake of GEN is related to potential health profits except learning and memory improvement (Adlercreutz et al., 1995; Zava and Duwe, 1997; Day et al., 2005).

Learning and memory is a unique brain function. It is mainly focused on the high degree of synaptic plasticity. Moreover, few natural extracts have an impact on the plasticity of central nervous system (CNS) (Sweatt, 2004; Feld et al., 2005; Giovannini, 2006; Ji et al., 2009; Peng et al., 2010). Furthermore, ERK1/2 belongs to mitogen-activated protein kinase family, which controls a variety of biological functions including learning and memory. In CNS, ERK1/2 regulates synaptic plasticity, neurogenesis, brain development and repair of nerve cells. Earlier studies suggested that GEN acts through ERK1/2 signaling and influences learning and memory(Zhu et al., 2001; Roberts and Der, 2007; Shioda et al., 2009; Alam and Gorska, 2011; Sun and Nan, 2017). However, GEN mediated learning and memory is not well established.

Further, long-term potentiation (LTP) is one of the cellular processes which are associated with learning and memory. In 2001, Dicristo et al. reported the contribution of cortical neurons in the LTP generation required for the activation of ERK signaling. Afterward Gooney et al. (2004) concluded that LTP is induced by brain derived neurotrophic factor (BDNF). Moreover, BDNF may be involved in the initiation of ERK1/2 signaling. Taken together, this paper aims to know the effect of GEN in the brain involving ERK1/2 and its manifestation in learning and memory of mouse.

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Material and methodology

Ethics

All the experiments related to animals were performed according to the guideline of Institutional Animal Ethical Committee (IAEC) of Indira Gandhi National Tribal University (IGNTU), Amarkantak, Madhya Pradesh, India, a central university established by the act of Govt. of India.

Animals

The *Swiss albino* male mice of 25weeks weighing 30 g were used for both the experiments *namely* behavioral analysis and immunoblotting. Mice were kept in polypropylene cages (3 mice/cage) under a 12/12 h light/dark cycle at an optimum temperature of 25 °C ± 1 °C with *ad libitum* access to water and food (obtained from Keval sales corporation, Vadodara, Gujarat, India).

Chemicals

All chemicals of analytical grade were used for the experiments. Basic chemicals were obtained from Loba, HiMedia, Merck and Sigma. PBS (Phosphate buffered saline), running buffer and transfer buffer, protein ladder MBT092-10LN, 6X protein loading buffer were obtained from HiMedia. Radioimmunoprecipitation assay buffer (RIPA) was prepared in the laboratory as per standard protocol and composition. Blocking buffer was prepared as per standard protocol in the laboratory. PVDF membrane TM PVDF (No. A10083099) was obtained from Amersham, GE healthcare life science. ECL was purchased from Advansta having catalogue no. K-12045- D20. Genistein was obtained from Otto chemiepvt ltd, Mumbai, India.

The chemical compositions of buffers used for the experiments were given as: 1XPBS (35 mM NaCl, 8 mM Na_2HPO_4,5 mM KCl, 7mMKH_2PO_4 (pH 7.6)/L, RIPA (50 mM Tris (pH 7.5), 150 mM NaCl, NP40 0.01%, 2 mM EDTA, 0.1%SDS, 1 mM PMSF, 0.05% (w/v) sodium deoxycholate/L. Blocking buffer (5% Non-fat dry milk in 1X PBS and 1X TBST/15 ml). X-Ray film developer and fixer were purchased from Bromex. Developer was prepared by dissolving 40.25 g in 450 ml water and fixer was prepared by dissolving 6.75 g in 450 ml.

Antibodies

The primary ERK1/2 antibody (Sc135900) and β3-tubulin (Sc80005) were obtained from Santa Cruz Biotechnology. The secondary goat anti mouse antibody (Invitrogen A10677) was obtained from ThermoFisher Scientific.

Experimental group and dosage

Male mice were placed in two groups; control and GEN treated (9 animals in each group). GEN was dissolved in sesame oil. The GEN dose was used 1 mg/kg/day as per Kohara et al. (2015) for the experiments. GEN was administered 24 h before each behavioral experiment. The concentration of GEN was 10 mg/1000 μl; 3 μl of this contains 0.03 mg GEN. From it, 3 μl was taken and volume was adjusted to 300 μl sesame oil. Finally, 0.03 mg of GEN was introduced by oral gavage tube to each mouse weighing 30 g.

Behavioral tests

To assess the effects of GEN treatment, mice were tested in OF and NOR. Habituation was performed before OF and NOR test.

Habituation

Habituation was performed for two days (once in a day). Briefly, before experiments every mouse was kept in OF for 5 min to make it familiar with the experimental instrument. OF was an open square box painted black from inside. Box was opened by upper side and covered by 40 cm high wall. Box size was 40 cmx40 cm. The movements of mice were recorded using camera (company name-Logitech, code-VU0019). After habituation, GEN was administered in treated group whereas sesame oil was administered in control group.

OFT (Open field test)

We analyzed motor function of mice using an OFT. OF was 40 cm X 40 cm square box. Box was divided into two parts namely outer squares and inner squares. The outer squares have sixteen squares adjacent to the outer wall denoted as protected fields. The inner squares have other nine squares denoted as exposed field. The test was started with the introduction of a mouse in the center of OF. Mouse was allowed to move generously in the box. Behavior of each mouse in the OF was recorded for 5 min. This entire process was followed for each mouse of both groups. After each test, the inner area of the box was sanitized with 70% ethanol and kept for 3 min to dry. Behavioral activity of mice from recorded video was analyzed by ANY-MAZE software (version 4.99).

NOR (Novel object recognition test)

NOR is a rapid and efficient method to test various stages of learning and memory of mice. NOR test comprises of three sessions: first habituation, second training and third test. Basically, training started with visual exploration of two identical objects, whereas the test session started swapping one of the formerly exposed objects with a novel object. In the present study, mice of both control and GEN treated groups were trained for two days with similar objects before performing learning and memory test using NOR. Thereafter, analyses of object recognition memory function were performed in the same open field. In NOR test, two identical objects by shape (square box), size and color (A and B) were kept in the box and mice were allowed to be familiar with these objects for 5 min. After that GEN was administered in mice using oral gavage. After 24 h of GEN treatment, mice were again kept in a box, and allowed to expose with dissimilar objects (object A was replaced with object C; object C was different by shape (round), size, and color from object A). Finally, behavioral activity of the mice was analyzed using ANY-MAZE software (version 4.99).

Protein preparation and immunoblotting

Protein from whole brain was prepared in RIPA buffer and estimated by Bradford method (1976). For IB, 30 μg protein was boiled at 100 °C with 6X loading buffer for 5 min followed by resolving in 10% SDS-PAGE. After resolving proteins, gel was kept in transfer buffer for 10 min. Then, PVDF membrane was dipped in absolute methanol for 30 s. Thereafter proteins on the gel were transferred onto PVDF membrane using semi dry apparatus. After that, membrane was incubated in 5% non-fat milk solution (blocking buffer) on a rocker for 4 h at room temperature. Thereafter, PVDF membrane was incubated with ERK1/2 primary antibody (1000X) at 4 °C overnight. Next day, the membrane was washed with 1X PBS followed by incubation in 2000X diluted secondary antibody dissolved in 1X PBS for 4 h at room temperature. Then the membrane was washed in 1X PBS for 5 min each (3 times). Finally, protein bands were detected using ECL method. The same methods were followed for detection of β3-tubulin band. Densitometric analysis of ERK1/2 and β3 tubulin signals was done using Image J software. The relative density value (RDV) was calculated by integrated density values of ERK1/2 divided by integrated density values of β3-tubulin of corresponding lane. The data were analyzed statistically using IBM*SPSS*-Statistics version 22, Java, 64-bit edition, 2013. It was followed by student *t*-test. Data with $p < 0.05$ were considered as significant.

Results

Open field test

In OFT, we did not find any significant change in the locomotor activity of mouse in GEN treated group in comparison to control (Fig. 1A, B, C).

Novel object recognition (NOR) test

In similar object test, no significant changes were observed in GEN treated mouse as compared to control (Fig. 2A, B, C, D). It means memory of mice of both groups is similar. However, in NOR test, we noticed that entry of mouse in both object B and C was increased; but entry in object C was more in GEN treated mouse in comparison to control (Fig. 3A, B, C,D).

Calculation of Discrimination Index (DI)

The relationship between the amount of exploration during the familiarization process and the corresponding object recognition memory can be tested using the NOR test. In the present study, DI was calculated on the basis of previous study (Antunes and Biala, 2012). DI value of present study for both groups, Control Group DI = [(16.3–19.133)/(16.3 +19.133)] = −0.079, GEN Group DI = [(32.3–26.46)/32.3 + 26.46)] = 0.099. DI permits distinction between

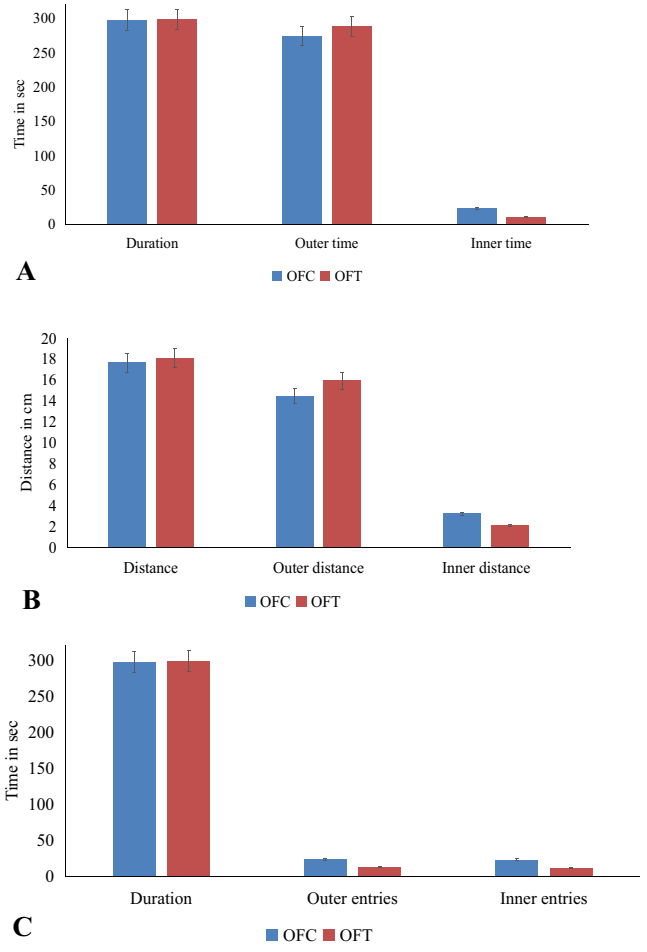

Fig. 1. Bar diagram of OFT of control and GEN treated mouse. X axis represents experimental parameters; duration, outer time, inner time ((A) Fig. 1A), distance, outer distance, inner distance ((B) Fig. 1B), duration, outer entries, inner entries ((C) Fig. 1C) respectively. Y axis denotes time spent in second by mouse in the open field arena (Fig. 1A and Fig. 1C) and distance in cm (Fig. 1B) respectively. Dark blue shows bar diagram of control group whereas dark maroon shows bar diagram of GEN treated group. Data were statistically analyzed using an independent student *t*-test. The p values < 0.05 were considered as significant. Abbreviation: *OFC (open filed control), OFT (open field treated).

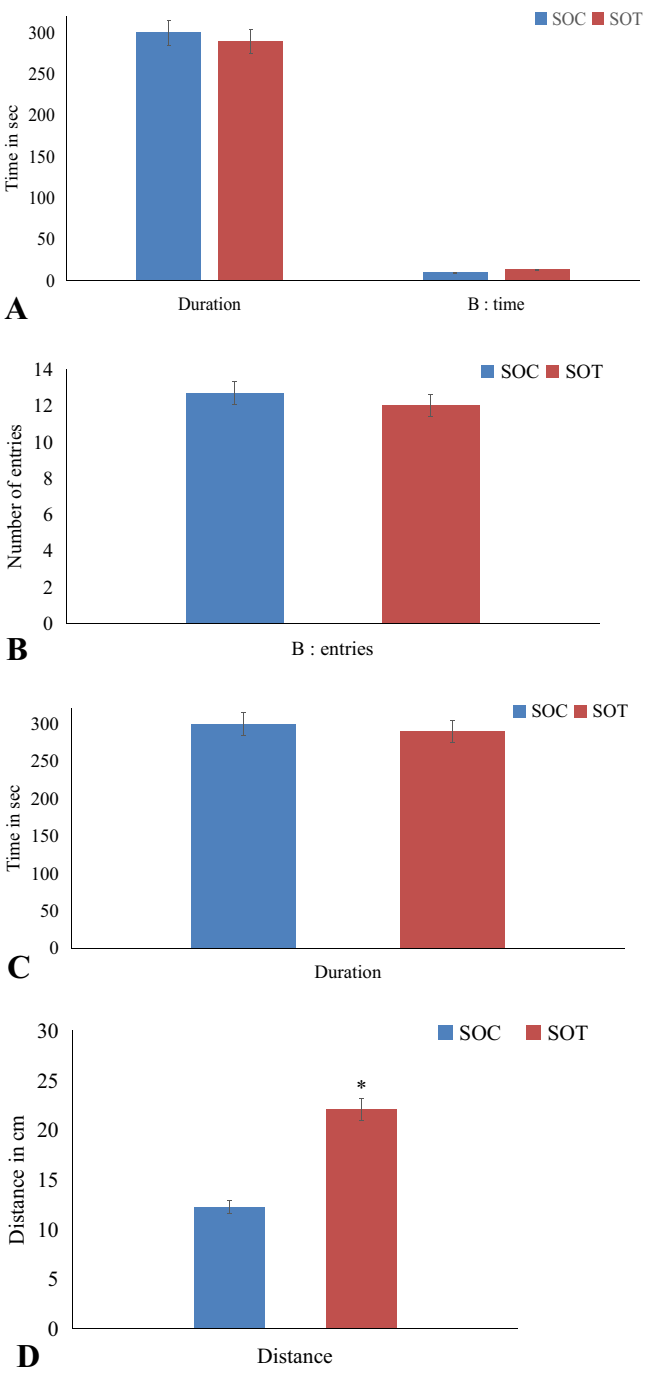

Fig. 2. Bar diagram of similar object test analysis of control and GEN treated mouse. X axis represents experimental parameters; duration and B time (A), B entries (B), duration (C), distance (D) respectively. Y axis denotes time spent in second by mouse in the open field arena (A and C), number of entries (B) and distance in cm (D) respectively. Dark blue shows bar diagram of control group and dark maroon shows bar diagram of GEN treated group. Data were statistically analyzed using an independent student *t*-test. The p values < 0.05 were considered as significant* . Abbreviation: SOC (similar object control) and SOT (similar object treated) * .

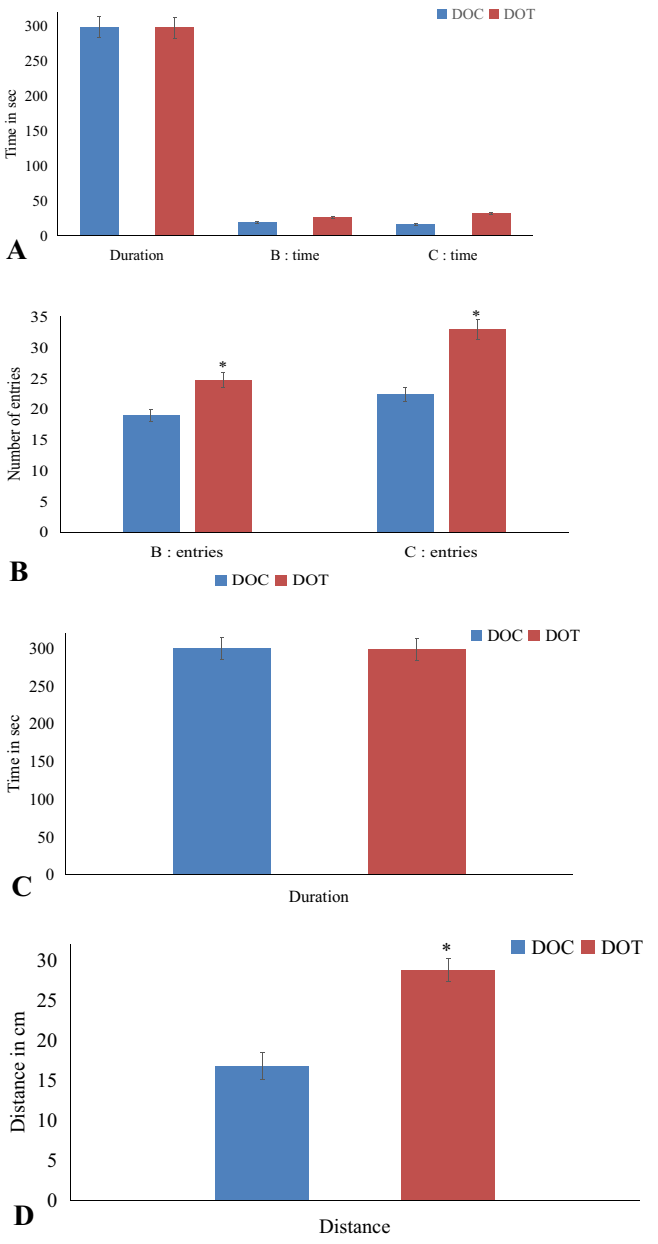

Fig. 3. Bar diagram of NOR analysis of control and GEN treated mouse. X axis represents experimental parameters; duration, B time and C time (A), B entries and C entries (B), duration (C) and distance (D) respectively. Y axis represents time in second spent by mice in the box (A and C), number of entries (B) and distance in cm (D) respectively. Dark blue is for control group and dark maroon is for GEN treated group. Data were statistically analyzed using an independent student *t*-test. The p values< 0.05 were considered as significant* . Abbreviation: DOC (dissimilar object control), DOT (dissimilar object treated).

novel objects and similar objects, i.e. measures the difference in exploring duration for common objects, but then divides this value by the total sum of exploring of novel objects and similar objects [DI = (T_N - T_F)/(T_N + T_F)]. The outcome of DI differs between + 1 and-1. The positive score suggests greater time spent on a novel object, a negative score indicates more time spent on a similar object, and a zero score indicates a null preference (Antunes and Biala, 2012).

Expression of ERK1/2 in mouse brain

Immunoblotting showed that expression of ERK1/2 was significantly higher in GEN treated group in comparison to control mouse brain

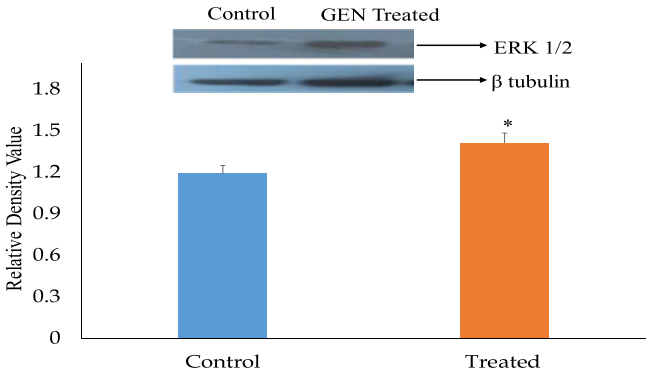

Fig. 4. Immunoblotting of ERK1/2 from control and GEN mouse brain. The upper panel shows immunoblot of ERK1/2 and β3 tubulin whereas in lower panel, X axis represents experimental group. Y axis represents relative density value. Light blue shows bar diagram of control group and light saffron shows bar diagram for GEN treated group. Data were statistically analyzed using an independent student *t*-test. The p values < 0.05 were considered as significant * .

(Fig. 4).

Discussion and conclusion

OFT and NOR test were used to evaluate the effects of GEN in anxiety, locomotor activity and learning and memory activity of mice. OF data suggested no changes in locomotor activity and anxiety of mice after GEN treatment in comparison to control (Malinowska et al., 2010; Rodríguez-Landa et al., 2017; Pierzynowska et al., 2019). Nevertheless, NOR data suggested that GEN treatment significantly increased learning and memory, especially retrieval process in GEN treated mouse in comparison to control (Pierzynowska et al., 2019). By taking both outcomes, our present study provides evidences that learning and memory capacity of mouse was increased due to GEN not by problem in locomotor activity and/or due to anxiety (Ball et al., 2010; Menze et al., 2015; Jiang et al., 2017).

Further, we also checked the effects of GEN on ERK1/2 expression, as ERK1/2 has been suggested to act as a key molecule in learning and memory. Immunoblotting showed significantly high expression of ERK1/2 in GEN treated mouse brain in comparison to control. ERK1/2 is a key signaling molecule that controls both the cell proliferation and stress (Zhai et al., 2013). There are numerous studies showing the significance of ERK1/2 mediated retrieval, its consequences, extinction and reconsolidation of memory. They suggested that the decrease of ERK1/2 stimulation prior to testing in 24 h or 48 h after training actually eliminated commencement of aversive and spatial memory (Besnard et al., 2013, 2014; Sindreu et al., 2007; Szapiro et al., 2000).

Furthermore, activation of ERK1/2 due to retrieval was confirmed through various learning tasks (Medina and Viola, 2018). For example, the activity of ERK1/2 was increased after inhibitory avoidance retention test in rats. This increase of ERK1/2 is relative to quantity of retrieval (Sindreu et al., 2007). Further, more studies have suggested the involvement of ERK1/2 for memory retrieval comprising of reconsolidation and extinction as primary consequences (Merlo et al., 2014, 2018). Briefly, reconsolidation means retrieval mediated LTM destabilization and/or stabilization includes post-translational modifications and modulation of gene expression. The role of ERK1/2 for reconsolidation of recognition memory in hippocampus has been reported earlier (Kelly et al., 2003). Moreover, impact of ERK1/2 signaling in appetizing learning tasks and specific aversive were reported. It indicates reconsolidation of memory in selected brain regions (Duvarci et al., 2005; Krawczyk et al., 2015; Miller and Marshall, 2005).

In addition, earlier studies suggested that ERK2 plays a decisive role in reconsolidation of fear conditioning (Cestari et al., 2006). Further, to

memory restabilization, ERK1/2 plays an important role in memory destabilization (Medina and Viola, 2018). In this line of work, Merlo et al.(2018) confirmed that the initiation of different ERK1/2 pools located in amygdala is NMDA receptor dependent. Moreover, these were necessary for fear reconsolidation as well as extinction. ERK/MAPK signaling has been found crucial for maintaining long-lasting memory storage in rodents. Nonetheless, BDNF dependent ERK1/2 activation has been found significant for the perseverance of two aversive learning tasks (Bekinschtein et al., 2008; Zhang et al., 2018). Zhang et al. (2018) have suggested that ERK signaling is a conserved mechanism and significant for memory determination. It is likely that GEN acts through ERK1/2 phosphorylation and improves learning and memory of mouse (Igaz et al., 2004). However, previous studies suggested that GEN might inhibit the LTP and ERK phosphorylation at higher concentration (O'Dell et al., 1991; Casey et al., 2002).

Moreover, another study showed that timing has an impact on maximizing hippocampal LTP. It predicts training regimens which offset defects in synaptic chemistry and memory in the fragile X mental retardation 1 (Fmr1) KO mouse model. In this experiment, wild-type mice took far less training to form stable memories when given three training trials separated by 1 h, opposed to one extended session; shorter or longer intervals. Interestingly, the same spaced training protocol rescued memory in Fmr1 KO mice and restored activation of synaptic ERK1/2, a kinase critical for both LTP and learning. This study provides support to our finding and suggests that the activation of ERK1/2 is important for learning and memory and ERK1/2 may get activated by various factors including GEN. Taken together, it could be interpreted that GEN enhances learning and memory of mouse involving ERK1/2. In addition, ERK1/2 has been reported to be involved in neurogenesis, synaptogenesis and LTP formation which influence learning and memory. Moreover, earlier study suggests that learning and memory depends on ERK1/2 expression and its phosphorylation (Cohen-Matsliah et al., 2010). Such study may be helpful to understand GEN mediated learning and memory involving ERK1/2.

CRediT authorship contribution statement

Khuleshwary Kurrey: Conceptualization, Methodology, Investigation, Formal Analysis, Writing- Original draft preparation and revising the manuscript. **Vijay Paramanik**: Conceptualization, Resources, Visualization, Project Administration, Supervision, Writing, Reviewing, Editing and revising the manuscript.

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Conflict of Interest

Authors declare no conflict of interest in any issue.

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